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Saliva-Exosomics in Cancer: Molecular Characterization of Cancer-Derived Exosomes in Saliva

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Abstract

Exosomes are small membrane vesicles of endocytic origin that are secreted by most cells and detected in saliva. Pathophysiological roles for salivary exosomes are beginning to be recognized in diseases including cancer, highlighting potential biomarkers and biological functions. Since early detection of cancer is vital for successful treatment, salivary exosomes would be advantageous in achieving a better survival rate due to their ready availability and noninvasiveness. The use of salivary exosomes may therefore be promising in the accurate detection of premalignant lesions and early-stage cancers, also for better our understanding of the molecular basis of tumorigenesis. In this chapter, we review our current knowledge of salivaomics, focusing on nucleic acids and proteins in saliva as potential cancer biomarkers. Since salivaomics is a rapidly evolving field, we hope to expand frameworks toward salivary exosomes, integrate new and existing information, and bridge salivaomics with other biomedical researches. Furthermore, we would like to coin the term “saliva-exosomics” as the next-generation salivaomics. Our goal in this chapter is to provide the most updated information on cancer-derived exosomes in the saliva as natural carriers of biomarkers and signaling molecules. Major advances include definitive structure analysis and molecular characterization of salivary exosomes. We also highlight the exosome biogenesis and cargo trafficking mechanisms in which recent animal studies have expanded our understanding of exosome-mediated transfer of cancer-derived products from distal tumor to salivary gland. The potential roles of the salivary exosomes in cancer progression and immune surveillance are also addressed.

1. INTRODUCTION

Saliva is a mixture of the secretions from the three pairs of major salivary glands (parotid, submandibular, and sublingual) and numerous minor salivary glands that have been

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DISCLOSURE STATEMENT

D.T.W.W. is the cofounder of RNAmeTRIX Inc., a molecular diagnostic company. D.T.W.W. holds equity in RNAmeTRIX and serves as a company Director and Scientific Advisor. The University of California also holds equity in RNAmeTRIX. Intellectual property that D.T.W.W. invented and which was patented by the University of California has been licensed to RNAmeTRIX. D.T.W.W. is a consultant to GlaxoSmithKlein, PeriRx, Wrigley, and Colgate-Palmolive.

identified throughout the oral mucosa [1]. The combination of these secretions including gingival crevicular fluid is called whole saliva, containing proteins (e.g., hormones, enzymes, cytokines, and antibodies), microorganisms, and cellular debris [2]. Saliva is a body fluid with many biological functions essential for food digestion and maintenance of oral health. Saliva plays critical role in the protection of teeth through buffering action and remineralization of enamel. The biological properties of saliva further support its important role in maintaining oral and systemic health through biological activities such as antibacterial and antiviral activity, which are associated with lactoferrin, lactoperoxidase, and immunoglobulin A secretion into saliva [3–5].

In the past decade, saliva researchers have been developing salivary diagnostics to detect oral and systemic diseases. Currently, saliva is an emerging biofluid for early detection of diseases, regarded as a mirror of oral and systemic health and a valuable source for clinically relevant information. In fact, several studies have demonstrated that saliva is useful for the detection and diagnoses of malignancies, human immunodeficiency virus, cardiac disease, and autoimmune diseases [6]. A wide variety of molecules are present in saliva, rendering saliva an attractive source of disease biomarkers. Saliva is composed of more than 99% water and less than 1% of other substances such as proteins/peptides, nucleic acids, electrolytes, and lipids that originate from multiple local and systemic sources [7,8]. Significant overlap between proteins detected in saliva and serum is due to the physical interaction between saliva and blood sources [9], indicating the potential of alternative approaches to examine systemic conditions. The recent proteomic studies of saliva confirmed that 20%–30% of the salivary proteome overlaps with the plasma proteome [9,10], indicating that the majority of salivary protein contents are synthesized in salivary glands [11], and the rest of the proteins are transported from blood or lymph into saliva [12]. These findings suggest that saliva is a particularly attractive fluid for biomarker research and not merely an adjunct to the blood, since saliva can itself provide unique information about diseases.

Importantly, saliva has advantages over blood as a body fluid for clinical diagnosis. For example, whole saliva collection is easy and noninvasive, thereby reducing patient discomfort when repeated sample collections are required. In addition, saliva does not coagulate, making it easier for handling and processing than blood. The development of particular saliva biomarkers and in-clinic small analyzers could facilitate diagnostics at the point of care [13]. However, despite these advantages over blood in terms of noninvasiveness and easy sample collection [6], there are still challenges that need to be addressed to establish salivary diagnostics. Saliva contains abundant amylase (approximately 60% of the total serum amylase) [14] and other proteins (e.g., proline-rich proteins) [15] that could potentially mask the presence of low levels of proteins, which might be the important biomarkers [16]. The composition and concentration of analytes in saliva are influenced by subject conditions and circadian rhythms [17]. Collection methods, when examining unstimulated vs stimulated saliva, or whole saliva vs individual gland saliva, are also significant parameters that influence the detection of biomarkers. The collection of unstimulated whole saliva, by swab or simply spitting into tube, is subject to considerable variation depending on the movements of tongue and cheek. Stimulated saliva can be collected by the use of an acid or gum to promote saliva flow [2] and yields three times the

volume of unstimulated saliva [18], although rapid flow rate and the duration of stimulation may affect saliva composition [19]. Whole saliva usually requires centrifugation or filtration to remove cellular contaminants regardless of collection methods since whole saliva contains cell debris and bacterial cells. However, bacterial contents still remain in the supernatant even after centrifugation [20], and filtration is not a perfect solution since it causes protein coagulation, affecting recovery and downstream experiments. The variable nature of whole saliva means that standardized methods have to be adopted to minimize variability and circadian fluctuations when studying saliva composition for the detection of disease biomarkers [21,22]. Several salivary studies have been conducted using unstimulated whole saliva [23–25] since it is easier to collect and may better reflect the status of systemic conditions [26]. We have performed proteomic analysis on whole saliva as well as ductal saliva obtained from the different glands, yielding important clues to the disease biomarkers [27,28]. However, the main barriers to more widespread adoption of salivary diagnostics are from the contamination and variable nature of saliva. For these reasons, we believe that the salivaomics should focus more on salivary exosomes (extracellular vesicles (EVs) in saliva) to overcome these limitations. Exosome and microvesicle subfractions will reduce the complexity of saliva and provide more stable and accurate clinically relevant information for disease detection. There are three main classes of EVs in saliva: exosomes, microvesicles, and apoptotic bodies [29–31]. Exosomes are derived from multivesicular bodies (MVBs), while microvesicles are generated by shedding from the plasma membrane [32]. Exosome constituents have been studied as potential diagnostic biomarkers for cancer, and many clinically important molecules (protein, RNA, and DNA) can be detected through various “-omics” approaches. Thus, the potential to detect secreted molecules directly from salivary exosomes in cancer patients can provide new opportunities in the development of noninvasive diagnostics.

2. SALIVAOMICS IN CANCER

2.1 Salivary Genomics in Cancer

Salivaomics is defined as the integrative study of saliva and its constituents, functions, and related techniques [33,34]. Salivaomics technologies have a broad range of applications in areas such as genomics, epigenomics, transcriptomics, proteomics, metabolomics, and microbiomics. In the past decade a number of findings have been generated using high-throughput technologies, prompting interest in the use of saliva as a source of biomarkers.

Saliva contains cell-free DNA, and genomic analysis revealed that 70% of salivary DNA originates from the host and 30% from the oral microbiota [35]. Salivary DNA is stable, and quality is relatively high [36–38], suggesting that salivary DNA is a useful target for the development of biomarkers. Circulating tumor DNA (ctDNA) is a cell-free DNA 180–200 base pairs in length, sheds from tumor cells into the circulatory system [39–44]. Tumor cells release DNA into the blood as either free DNA or tumor-derived exosomes by mechanisms not well understood [39,45–48]. ctDNA has been detected in various bodily fluids, including blood, urine, and saliva [49,50]. ctDNA can be distinguished from normal cell-free DNA by the presence of mutations, and clinical testing of ctDNA is referred to as liquid biopsy [51,52]. ctDNA has been studied as a promising biomarker in an increasing number of

cancer types, and now several clinical trials are ongoing [48]. Because conventional tissue biopsy only provides partial information of heterogeneous tumors at one time [53–58], liquid biopsy has the potential to detect and monitor tumor progression in real time with more accuracy [39,45–48,55,57]. Detection technologies must be highly sensitive since ctDNA comprises of only a minor fraction of the total cell-free DNA. The most common technologies for ctDNA detection are next-generation sequencing and digital PCR [46,47]. However, simple, compact, and more cost-effective analyzers with high levels of sensitivity and specificity are desirable to facilitate routine clinical care. Recently, we developed a novel salivary diagnostics platform EFIRM (electric field-induced release and measurement) that can detect and quantify ctDNA with high sensitivity using electrochemical sensor [50]. We tested the ability of EFIRM to detect tyrosine kinase inhibitor-sensitive *EGFR* mutations (exon 19 deletion and L858R mutations) directly from saliva and plasma of lung cancer patients [50,59]. A receiver-operating characteristic (ROC) curve analysis demonstrated that EFIRM detected *EGFR* exon 19 deletion and L858R mutation in saliva with an area under the ROC curve (AUC) of 0.94 and 0.96, respectively, suggesting that saliva-based *EGFR* mutation detection (SABER) met the clinical requirements.

2.2 Salivary Transcriptomics in Cancer

Transcriptome is the complete set of RNA molecules, including mRNA, microRNA (miRNA), piwi-interacting RNA (piRNA), and other small RNAs such as rRNA and tRNA. Salivary transcriptomics has emerged as a powerful approach for exploring salivary biomarkers and focuses mainly on mRNA and miRNA [60]. Although the genome is the same in all host cells, different cells and body fluids show different patterns of RNA composition. Therefore, transcriptomic analysis can provide valuable information about disease states. In 2004, the human salivary transcriptome was first discovered in our laboratory using microarray technology [61]. We characterized salivary transcriptome as highly fragmented coding and noncoding gene transcript derived from host and oral microbiota [60,62,63]. We also developed a method that allows stable and direct saliva transcriptomic analysis without further processing [64,65].

We analyzed the salivary transcriptome in cancer patients in attempts to improve early diagnosis. Our prior transcriptomic studies have discovered seven mRNA biomarkers (*DUSP1*, *H3F3A*, *IL1B*, *IL8*, *OAZ1*, *S100P*, and *SAT*) in saliva samples from patients with oral squamous cell carcinoma (OSCC) [66]. A logistic regression model including four markers (*IL1B*, *OAZ1*, *SAT*, and *IL8*) achieved 91% sensitivity and 91% specificity (AUC = 0.95). We further validated these oral cancer biomarkers using an additional two independent cohorts and demonstrated that AUC for prediction of OSCC ranges from 0.74 to 0.86 across the five cohorts [67]. These findings suggested that salivary mRNA can be potential biomarkers for oral cancer detection and opened a new avenue for salivary biomarker discovery for other cancers. Since 2010, we have reported salivary mRNA biomarkers in various cancers. We have found four salivary transcriptomic biomarkers (*KRAS*, *MBD3L2*, *ACRV1*, and *DPM1*) associated with resectable pancreatic cancer [68]. A logistic regression model demonstrated that the combination of four markers could differentiate cancer patients from control subjects with 90% sensitivity and 95% specificity (AUC = 0.971). The utility of salivary mRNA was further demonstrated for the detection of breast cancer [69], ovarian

cancer [70], and lung cancer [71]. We profiled salivary transcriptomes and proteomes of breast cancer patients [69]. Preclinical validation demonstrated that the combination of eight transcriptomic bio-markers (*CSTA*, *TPT1*, *IGF2BP1*, *GRM1*, *GRIK1*, *H6PD*, *MDM4*, and *S100A8*) and one proteomic biomarker (CA6) could discriminate between breast cancer and control group with 83% sensitivity and 97% specificity. Transcriptomic analysis of saliva in ovarian cancer identified 4 upregulated and 16 downregulated genes [70]. The logistic regression model demonstrated that the combination of five biomarkers (*AGPAT1*, *B2M*, *IER3*, *IL1B*, and *BASP1*) showed 85.7% sensitivity and 91.4% specificity. Furthermore, we found significant differences in salivary mRNA between patients with lung cancer and healthy controls [71]. A logistic regression model using the combination of five markers (*CCNI*, *FGF19*, *GREB1*, *FRS2*, and *EGFR*) could differentiate lung cancer from control with 93.75% sensitivity and 82.81% specificity (AUC = 0.925). Hence, salivary mRNA biomarkers could be useful for cancer screening.

Detection of miRNA from body fluids is also becoming increasingly important in liquid biopsy. Many studies have shown that miRNAs are frequently dysregulated in cancer [72]. Therefore, alterations in miRNA expression patterns often correlate with disease and can be promising diagnostic biomarkers for cancer. Importantly, salivary miRNAs are more stable [73,74] and discriminatory [75,76] than salivary mRNAs. Thus, circulating miRNAs are attractive potential biomarkers. In 2009, we discovered miRNAs in saliva and characterized them as possible biomarkers for oral cancer detection [74]. Two miRNAs (miR−125a and miR−200a) were significantly reduced in the saliva of oral cancer patients compared to control group. Furthermore, in the validation study for salivary gland tumors, we demonstrated that combination of four miRNAs (miR−132, miR−15b, miR−140, and miR −223) can distinguish between malignant and benign parotid gland tumor with 69% sensitivity and 95% specificity (AUC = 0.90) [77].

More recently, we reported piRNA in saliva as an emerging potential biomarker for cancers [78]. We conducted high-throughput sequencing of piRNA in human saliva from healthy individuals, showing comparable expression patterns and levels compared to that in other body fluids. These findings opened the doors for further investigation of salivary piRNA as cancer biomarkers.

2.3 Salivary Proteomics in Cancer

Comprehensive analysis of the salivary proteome is critical for appreciating its full diagnostic potential. A consortium of three research groups including The Scripps Research Institute, UCSF, and our laboratory at UCLA has compiled a comprehensive catalogue of the salivary proteome of healthy individuals, identifying 1166 proteins in parotid and submandibular/sublingual gland ductal saliva [27]. The data set from this study has been deposited into the Saliva Proteome Knowledge Base (http://www.skb.ucla.edu/cgibin/spkbcgi-bin/main.cgi) for public access [33]. Bandhakavi et al. made a significant contribution to expand our understanding of the salivary proteome by using three-dimensional peptide fractionation and generated the largest whole saliva proteome dataset including 2340 proteins that are involved in a variety of biological functions in the oral cavity [10]. Comparative analysis of human saliva and plasma proteome showed that

distributions of salivary proteins are enhanced in two gene ontology categories (metabolic and catabolic processes) compared with plasma, suggesting that saliva may be advantageous over plasma, especially for less abundant proteins involved in these biological processes [79]. Unlike serum proteins, salivary proteins appear to be more susceptible to degradation [80,81]. Esser et al. reported that salivary proteins can degrade rapidly even during saliva collection and handling, which may hamper the downstream experiments and application [82]. We have established methods to stabilize salivary proteins using protease inhibitors, thereby enabling us to store saliva samples for up to 2 weeks without significant degradation [83].

To our knowledge, the first attempt at cancer diagnosis using salivary protein was made by Hoerman et al. more than 50 years ago. They showed that patients with prostate cancer exhibited elevated acid phosphatase enzymatic activity in parotid saliva [84]. In the past decades, there has been a marked advancement in protein analytical technologies combined with bioinformatics, creating a new revolution in salivary proteomics. Currently, high-throughput mass spectrometry is the core technology for salivary protein identification, and several salivary proteins were identified as potential salivary biomarkers for the detection and monitoring of cancer (Table 1).

2.3.1 Breast Cancer—Breast cancer is the most well-studied cancer in salivary proteomics. Significantly elevated EGF [85], c-erbB-2 [86,87], and CA15–3 [87] were identified in the saliva of breast cancer patients compared to noncancer groups. There was a significant positive correlation between serum and saliva CA15–3 level, indicating the potential of saliva as a diagnostic body fluid for breast cancer [98,99]. While serum CA15–3 is now an FDA-approved biomarker for monitoring the metastasis of breast cancer [100], salivary CA15–3 has not been cleared for this application by FDA. We analyzed VEGF, EGF, and CEA in saliva from breast cancer patients to evaluate the predictive power of each protein individually and in combination [88]. A logistic regression model revealed that the best combination was salivary VEGF and EGF with 83% sensitivity and 74% specificity (AUC = 0.84). Furthermore, we identified CA6 and psoriasin as potential salivary biomarkers for breast cancer and then independently validated them using an independent cohort [69]. The results showed a significant difference in salivary CA6 between breast cancer and healthy controls ($P = 0.0017$). Recently, Wood et al. analyzed the concentrations of lung resistant protein (LRP) in saliva of Stage I breast cancer patients [89]. The results demonstrated that the saliva of the patients had significantly higher levels of LRP as compared to healthy controls, suggesting it is a novel biomarker for breast cancer.

2.3.2 Squamous Cell Carcinoma—Our prior salivary proteomic study discovered five candidate markers (M2BP, MRP14, CD59, catalase, and profilin) associated with OSCC [90]. A logistic regression model including five markers achieved 90% sensitivity and 83% specificity with cross-validation prediction accuracy rate of 85% (AUC = 0.93). Furthermore, by using the additional two independent cohorts, we validated the previously identified three salivary protein markers for oral cancer (IL-8, M2BP, and IL-1B; [66,90]). We found that IL-8 and M2BP were significantly different ($P<0.02$) and that IL-1B was marginally different ($P = 0.053$) between cancer and control groups [67]. Ohshiro et al.

analyzed whole saliva from three head and neck SCC (HNSCC) patients using LC-MS/MS [22]. Among 34 proteins detected in saliva from NHSCC, alpha-1-B-glycoprotein (A1BG) and complement factor B (CFB) were identified as unique proteins in cancer. Dowling et al. analyzed whole saliva samples from HNSCC patients using 2D DIGE analysis and subsequent mass spectrometry [16]. The result identified that five proteins, including beta fibrin (FB), S100 calcium-binding protein (S100), transferrin (TF), immunoglobulin heavy chain constant region gamma (IGHG), and cofilin-1 (CFL1), were significantly increased in the saliva from HNSCC patients compared to the control group. Rai et al. assessed adenosine deaminase activity (ADA) in saliva of tongue cancer and found statistically significant differences between cancer and control groups, suggesting that ADA measurement might be useful as a diagnostic tool for early detection of tongue cancer [91].

2.3.3 Gastric Cancer—Much effort has been made to develop serum or plasma biomarkers for gastric cancer, and over 2000 studies were reported so far. The most commonly used diagnostic biomarkers are CEA and CA19–9. However, these biomarkers are not widely acknowledged in early detection of cancer because of their limited sensitivity (<21% sensitivity). The combination use of CEA, CA19–9, CA125, and AFP is suggested to improve sensitivity for the diagnosis of gastric cancer [101]. Despite enormous effort in serum biomarker discovery, there are only a few studies related to salivary protein biomarkers for detection of gastric cancer. Wu et al. identified four salivary proteins (1472.78, 2936.49, 6556.81, and 7081.17Da) by mass spectrometry and found that these proteins were significantly different between gastric cancer and normal groups [93]. Recently, we identified and quantified 519 proteins in the saliva of gastric cancer patients using mass spectrometry with tandem mass tag, among which 48 showed significant differential expression profile between cancer and controls [94]. Six proteins (CSTB, TPI1, DMBT1, CALML3, IGH, and IL1RA) were selected for initial verification by ELISA and found to be downregulated in the saliva of gastric cancer patients. A logistic regression model using three biomarkers (CSTB, TPI1, and DMBT1) in the prevalidation sample set resulted in 85% sensitivity and 80% specificity (AUC=0.930). These findings provided the proof of concept of salivary protein biomarkers for the noninvasive detection of gastric cancer.

2.3.4 Lung Cancer—We investigated salivary proteomic biomarkers in lung cancer patients and found that the levels of three proteins (HP, AZGP1, and CALPR) were significantly higher in lung cancer patients than healthy controls [95]. A logistic regression model including the three proteins achieved 88.5% sensitivity and92.3% specificity (AUC = 0.90), suggesting that salivary protein biomarkers have the potential for the detection of lung cancer.

2.3.5 Ovarian Cancer—Chen et al. analyzed CA125 in saliva samples from ovarian cancer patients and showed significant differences in salivary CA125 levels between the malignant and benign groups with 81.3% sensitivity [97]. There was a linear correlation between salivary and serum CA125 levels, suggesting the promising utility of saliva for monitoring ovarian cancer.

3. SALIVA-EXOSOMICS: NEXT-GENERATION SALIVAOMICS

3.1 Discovery of Salivary Exosomes

EVs are classified into three subgroups (exosomes, microvesicles, and apoptotic bodies) based on their size and biogenetic pathways [102,103]. Exosomes are intraluminal vesicles that are formed within MVBs and released upon the fusion of MVBs with the cell membrane [104]. Over 30 years ago, two independent groups observed that MVBs in reticulocytes released small vesicles into the extracellular space [105,106]. Then, the term “exosome” was first coined in 1987 by Johnstone et al. to describe that vesicles shed from cultured cells retained enzymatic activity as a remnant of the parental cells [107]. It was later determined that exosomes are EVs of endosomal origin that are not degraded by lysosomes, noting that exosome secretion is a way to excrete unnecessary products from the cells [108–110]. With the discovery that exosomes contain RNA, exosomes acquired substantial interest as mediators of cell-to-cell communication [111]. Exosomes have been isolated from most cell types [112] and body fluids such as blood [113], urine [114], saliva [115], breast milk [116], amniotic fluid [117], and semen [118,119]. Salivary exosomes were first described in 2008 when Ogawa et al. found that 30–130nm vesicles were present in human whole saliva [115]. The stability of exosomes in the circulation and body fluids has made exosomes attractive as potential biomarkers. Typically, exosomes are defined as vesicles ranging from 30 to 100nm in size and 1.13–1.19g/mL in density and are isolated through density gradient or sucrose cushion by ultracentrifugation at 100,000 × g [120]. However, the term “exosome” is now often used in a less restrictive manner than Johnstone’s original definition [121]. Also, exosome is a confusing term as it has been used to refer to exonuclease in cells [122]. In this chapter, we respect authors’ choice of vesicle nomenclature as long as it follows logical interpretation of the data. However, we carefully use the term “exosome” since a consensus on its origin remains elusive. For the nomenclature and definition of EVs, see the website of International Society for Extracellular Vesicles (http://www.isev.org), the American Society for Exosomes and Microvesicles (http://www.asemv.org), and Vesiclepedia (http://microvesicles.org).

Human saliva has been successfully demonstrated as an ideal fluid with distinct advantages for oral cancer diagnosis [67]. Exosome isolation from saliva has been optimized [123,124], and the use of this small but highly informative fraction may reduce the overall complexity of saliva as a result of contribution from local and systemic sources [125]. For these reasons, the study of vesicles secreted by cancer cells via MVBs into saliva could be an interesting clinical approach for the detection of novel biomarkers in cancer. Here, we first coin the term “saliva-exosomics” to describe next-generation salivaomics that studies salivary exosomes through the application and integration of advanced “-omics” technologies to better delineate their specific functions and for use as a source of noninvasive biomarkers for disease diagnosis.

3.2 Contents of Salivary Exosomes

Exosomes are surrounded by a phospholipid bilayer and carry a unique cargo of proteins and nucleic acids that can reflect those of the cell of origin (Fig. 1). The most commonly detected proteins are tetraspanins (e.g., CD63, CD9, and CD81), heat shock proteins (e.g.,

Hsp70 and Hsp90), major histocompatibility complexes (MHC class I and II), membrane transporters and fusion proteins (e.g., Rab GTPases and annexins), and ESCRT (endosomal sorting complex required for transport)-associated proteins (e.g., Alix and Tsg101) that are involved in exosome biogenesis from MVBs [126–129]. Other proteins found on exosomes include signaling, cytoskeletal, metabolic, and carrier proteins. Of note, MHC class I is ubiquitously expressed on all exosomes, while MHC class II is confined to exosomes derived from antigen-presenting cells, including dendritic cells, macrophages, and B cells [130]. Numerous proteomic studies on mammalian EVs have yielded extensive catalogues of proteins found in various types of EVs isolated from cells, tissue, or body fluids. The databases are publicly available at Vesiclepedia (http://www.microvesicles.org) [102] and ExoCarta (www.exocarta.org) [131]. Both databases include data not only on proteins but also on nucleic acids and lipids, as well as on the constituents isolated from salivary exosomes.

The first comprehensive proteomic analysis of human salivary exosomes was performed by Gonzalez-Begne et al. using a multidimensional protein identification technology [132]. The analysis identified 491 proteins in the exosome fraction of human parotid saliva, and Gene Ontology analysis found that cytosolic proteins comprise the largest category of the proteins. Comparison between the parotid salivary exosomes (491 proteins) and the previously identified global parotid saliva proteome (914 proteins; [27]) showed a 23% overlap between parotid salivary exosomes and parotid saliva, whereas 20% were unique to parotid exosomes and 57% were unique to parotid saliva. Of note, the water channel associated with the salivary secretion mechanism (aquaporin 5) was identified in salivary exosomes, reflecting the unique characteristics of the exosomes secreted by salivary gland (Fig. 1). Ogawa et al. revealed the presence of two different types of vesicles (exosomes I and II) with different mean diameter (I: 83.5nm, II:40.5nm) and protein constituents [133]. Overall, 101 and 154 proteins were identified in exosomes I and II, respectively, and 68 proteins including common markers (CD63, Alix, Tsg101, and Hsp70) were overlapped between the two groups. Approximately 40% of the proteins identified were extracellular (e.g., immunoglobulin chain) or secretory proteins (e.g., serum albumin), indicating that saliva contains vesicles originating from circulating lymphocytes and intravascular fluid. The presence of DPP IV (CD26) in the vesicles and its enzymatic activity was also demonstrated by showing DPP IV-dependent degradation of substrates (substance P and GIP), suggesting that the vesicles were biologically active [115]. We performed the proteomic profiling of salivary microvesicles with an average diameter of 100–1000nm to investigate a different type of EVs in saliva [134]. LC-MS/MS combined with gel electrophoresis identified 63 proteins in salivary microvesicles. Among 63 proteins identified in microvesicles, 35 proteins were exclusively identified in microvesicles by comparing them to parotid exosome proteome (491 proteins; [132]), suggesting that salivary microvesicles contained their own unique proteins. The most striking finding was that saliva triggers factor VII-mediated coagulation of human plasma [135]. Berckmans et al. tested the ability of saliva to induce clot formation of autologous plasma and found that salivary exosomes shortened the clotting time of exosome-depleted plasma. Western blot analysis and transmission electron microscopy (EM) with immunogold labeling identified tissue factor (TF), the initiator of coagulation activation, in salivary exosomes. Moreover, the salivary exosome-induced

shortening of the clotting time was completely abolished in the presence of anti-factor VII. These results indicated that saliva facilitates hemostasis as one of the first steps in the process of wound healing.

Salivary exosomes are also known to contain mRNA [136] and small RNA [123], including miRNA [73,124], piRNA, small nucleolar RNA (snoRNA), and other small RNAs (rRNA and tRNA) [137,138] (Fig. 1). Interestingly, piRNA appears to be found at higher abundance in exosomes compared to whole saliva [137,138]. We conducted high-throughput RNA sequencing (RNA-Seq) using human cell-free whole saliva [78] and found that the most abundant types of small RNAs were piRNA (7.5%), miRNA (6.0%), and snoRNA (0.02%), consistent with the prior study [137]. Exosomes offer protection from RNase [139], thereby inhibiting RNAs from degradation and allowing them to be taken up by the cells with subsequent putative effects on gene expression in the target cells [111,140]. Indeed, prior studies have shown that salivary exosomal RNAs are stable [136,141] and can be taken up and translated by recipient cells [123,136], indicating that these RNAs are biologically functional. Further salivaexosomics studies will provide important insights regarding the mechanisms that control epithelial cell homeostasis in the oral cavity.

3.3 Structure of Salivary Exosomes

Exosome characterization typically includes morphological analysis. Due to their nanoscale size, morphological analysis of exosomes had solely been limited to EM [142]. While EM has been a standard technique for exosome characterization, this technique may not provide a representative view for exosomes due to harsh sample processing such as fixation. Indeed, transmission EM has showed a cup-shaped appearance, while cryo-EM showed the round shape of the exosomes [31,143]. We first applied atomic force microscopy (AFM) and field emission scanning electron microscopy (FESEM) to assess the native salivary exosome structure and substructural organization unresolvable in transmission EM [136,144]. We found the distinct substructure of single isolated 70–100nm human salivary exosomes in the form of trilobed structures and demonstrated their reversible elastic nanomechanical properties. High-resolution AFM images correlated well with the exosome structures obtained from FESEM, where low force imaging resulted in round-shaped exosomes, suggesting exosomes have spherical morphology unless outside force is exerted on them. In addition, AFM phase contrast images of salivary exosomes indicated a heterogeneous surface, which was attributable to the presence of proteins in the highly dense lipid membrane, consistent with previous proteomic analysis of salivary exosome [132].

Salivary exosomes are naturally occurring bioparticles, parts of which are directly secreted from the surfaces of oral epithelial cells into saliva. Because exosomes released by normal and tumor cells have been suggested to differ in both functional and structural properties [145], oral cancer-derived exosomes in saliva have great potential as biomarkers for cancer diagnosis. To elucidate the structural differences between the salivary exosomes derived from healthy subjects and oral cancer patients, Sharma et al. investigated morphological characteristics at single-vesicle level using high-resolution AFM [146]. AFM imaging displayed irregular morphologies and higher intervesicular aggregation in cancer exosomes than normal exosomes. Quantitative analysis revealed that size and CD63 surface density

were significantly increased in cancer exosomes (98.3 ± 4.6 nm)compared to normal exosomes (67.4 ± 2.9nm) (P<0.05). Structural and morphological aberrations in the exosomes indicated that these exosomes are at least in part cancer-derived products that were directly shed into saliva. Interestingly, MVBs were identified in oral cancer salivary exosome fractions. These multivesicular structures revealed the presence of ruptures and elongated nanofilaments around the lumen of these MVBs, suggesting that these are the sites for exosome release as well as filamentous extension of nucleic acids (Fig. 2).

4. SALIVARY EXOSOMES AS POTENTIAL BIOMARKERS FOR CANCER

A breakthrough in the field of EVs was made in 2008 when it was discovered that glioblastoma microvesicles carry cancer-specific mutant mRNA (EGFRvIII) [147]. Strikingly, the tumor-specific EGFRvIII was detected in the serum microvesicles from glioblastoma patients, suggesting that tumor-derived microvesicles may serve as potential cancer biomarkers. Since its discovery, cancer-derived exosomes have been recognized as an important diagnostic tool. A series of “-omics” approaches has revealed that exosomal miRNA, DNA, and protein signatures may also serve as biomarkers to aid diagnosis. Serum exosomal miR−21 and miR−141 levels are significantly increased in the patients of esophageal SCC and prostate cancer, respectively [148,149]. Mutated KRAS and p53 DNA were detected by whole genome sequencing in the serum exosomes of pancreatic cancer patients [150], and the membrane-anchored exosomal protein glypican-1 (GPC-1) was identified as a promising biomarker of pancreatic cancer [151]. These findings clearly indicate that serum exosomes in cancer patients have great potential for cancer diagnosis.

We have focused our efforts on understanding the mechanisms and roles of salivary exosomes in cancer. To provide proof of concept for the potential utility of salivary exosomes for cancer detection, we developed a pancreatic cancer mouse model in which a mouse pancreatic cancer cell line (Panc02) was orthotopically injected into the pancreas of the syngeneic mice [152]. We investigated the role of pancreatic cancer-derived exosomes in salivary biomarker development by inhibiting the exosome biogenesis in Panc02 cells. Stable transfection of the dominant negative form of the GTPase Rab11 (DN-Rab11) effectively inhibited the biogenesis of exosomes in Panc02 cells and resulted in the ablation of discriminatory salivary biomarker signatures between tumor-bearing mice and control mice, suggesting that the observed changes in the salivary transcriptome were the result of the changes in cancer-derived exosomes. This study demonstrated that cancer-derived mRNAs are the cargo of exosomes and reach the salivary gland via circulation, providing a mechanistic link between discriminatory salivary biomarkers and distal tumor (Fig. 3). To further our understanding of the link between salivary exosomes and distal tumors, we generated a xenograft lung cancer mouse model in which H460 human lung cancer cells that stably express hCD63-GFP were orthotopically injected into immunocompromised mice [153]. We identified human GAPDH mRNA in hCD63+GFP+ exosome-like microvesicles in mouse saliva, indicating that exosome-like microvesicles carry tumor cell-specific mRNA and travel to the circulation and reach the saliva, where they have a potential role as tumor biomarkers. Our most recent animal study demonstrated the role of salivary exosomes in immune surveillance [154]. To investigate an immunoregulatory effect of salivary exosomes, saliva from Panc02-bearing mice was orally administered to nontumor-bearing control mice.

Expression levels of NK activation markers, CD69 and NKG2D, were significantly decreased by gavage feeding of tumor saliva, while these effects were ablated by DN-Rab11-mediated inhibition of exosome biogenesis in Panc02 cells. These animal studies supported our hypothesis that cancer-derived exosomes provide a rationale for the development of salivary biomarkers that are applicable to distal tumors.

Winck et al. performed a proteomic analysis of salivary EVs from OSCC patients and healthy controls, and a total of 381 proteins were identified in the EVs from the two groups by mass spectrometry [92]. Among the proteins identified in salivary EVs, eight proteins including alpha-2-macroglobulin (A2M), haptoglobin alpha chain (HPA), mucin-5B (MUC5B), galectin-3-binding protein (LGALS3BP), immunoglobulin alpha-1 chain c region (IGHA1), prolactin-inducible protein (PIP), pyruvate kinase isozymes M1/M2 (PKM1/M2), and GAPDH were differentially expressed between the two groups (ANOVA, $P<0.05$). Gene Ontology analysis showed that the salivary EV proteome in OSCC patients was enriched in proteins related to “molecular transport” and “cellular growth and proliferation,” suggesting that the properties of salivary EVs may reflect the tumor of origin. Sun et al. analyzed the proteome of salivary EVs isolated from lung cancer patients and identified 113 and 95 proteins in cancer patients and healthy controls, respectively [96]. Among the total 113 proteins identified in cancer group, 63 proteins were exclusively detected in the lung cancer group. The literature survey showed that 12 proteins are lung cancer-related biomarkers, including annexin family members (annexin A1, A2, A3, A5, A6, A11), nitrogen permease regulator 2-like protein (NPRL2), carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), histone H4 (HIST1H4A), mucin 1 (MUC1), prominin-1 (PROM1), and tumor necrosis factor alpha-induced protein 3 (TNFAIP3). Thus, these works open up promising new lines of research that may lead to the identification of new classes of cancer biomarkers. Further functional studies of this new player (salivary exosome) will provide new clues to its mechanism of action and simultaneously raise fundamental questions about the coregulation of serum and salivary exosomes on the progression of cancer.

5. FUTURE PERSPECTIVE

In the past decade, salivaomics studies have revealed the utility of the saliva in identifying the presence of cancer. As described here, much progress has been made in understanding characteristics of saliva, with significant advances on how the salivary constituents relate to their biomarkers and functions. In addition, a growing body of saliva-exosomics study is highlighting the role of cancer-derived exosomes in saliva. The unique properties of cancer-derived exosomes in saliva, which originate from organelles and are transferred into saliva, are attracting the attention of scientists since these exosomes could be used as diagnostic biomarkers, potential surrogate markers for other physical conditions, or novel immune regulatory systems through the gastrointestinal tract. However, the utility of salivary exosomes as biomarkers of diseases and conditions requires further investigations due to the current paucity of studies in this emerging area. Key future tasks will be validating salivary exosome biomarkers and determining the molecular mechanisms of exosome interaction between distal tumors and salivary glands. Also, establishing rapid and sensitive technologies to purify and analyze exosomes will represent an important future challenge.

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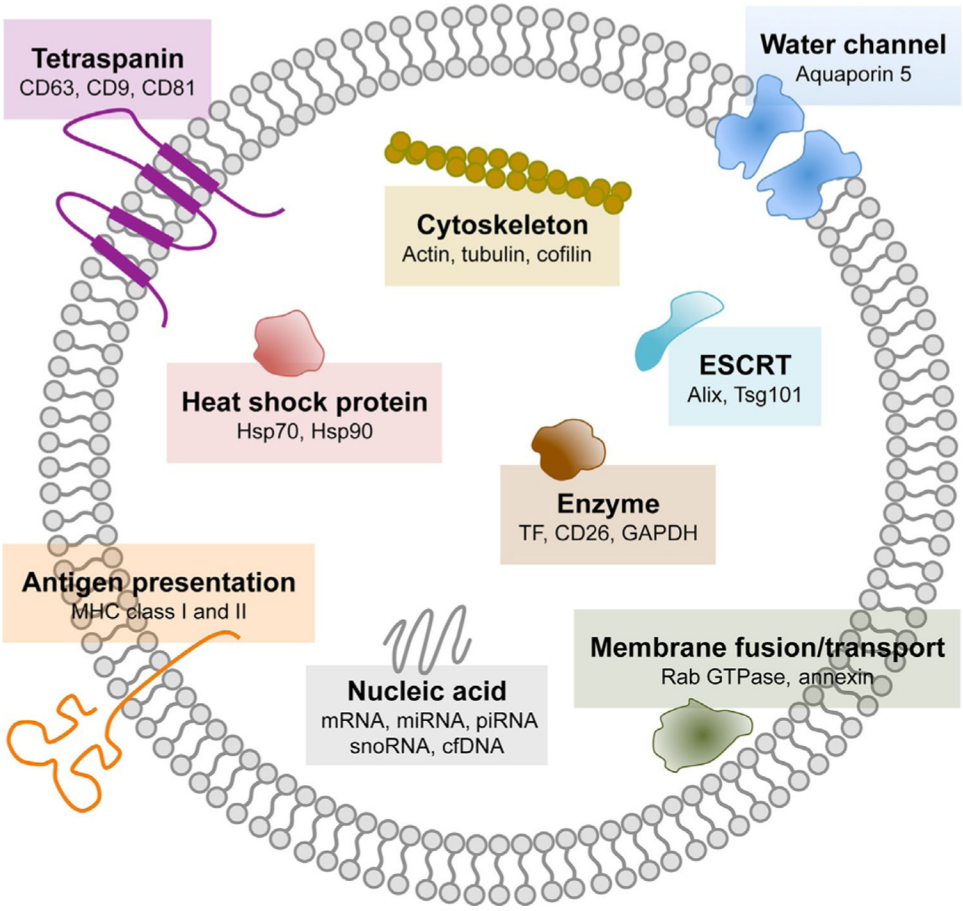

Fig. 1.

Structure and content of salivary exosome. Exosome is surrounded by a phospholipid bilayer. Membrane protein markers in most salivary exosomes include tetraspanin, water channel, MHC class I and II. The intravesicular contents include nucleic acids (RNA and DNA) and various cytosolic proteins such as enzyme, heat shock protein, cytoskeleton, ESCRT-associated proteins, and membrane fusion/transport-associated proteins from parental cells of origin. ESCRT, endosomal sorting complex required for transport; MHC, major histocompatibility complex; TF, tissue factor.

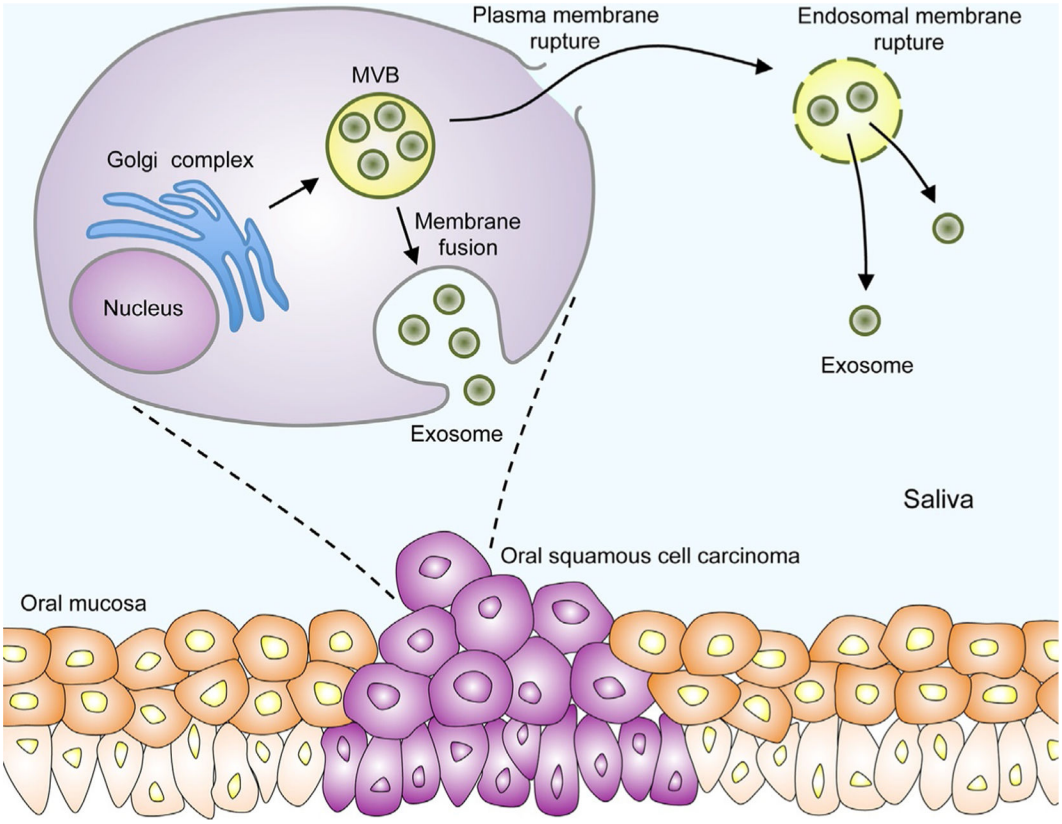

Fig. 2.

Exosome biogenesis in oral squamous cell carcinoma (OSCC) and direct release into saliva. Exosomes are generated via the trans-Golgi network and accumulated in MVB. Exosomes are released into saliva by two different mechanisms: constitutive release via membrane fusion or aberrant release via membrane rupture. MVB, multivesicular body.

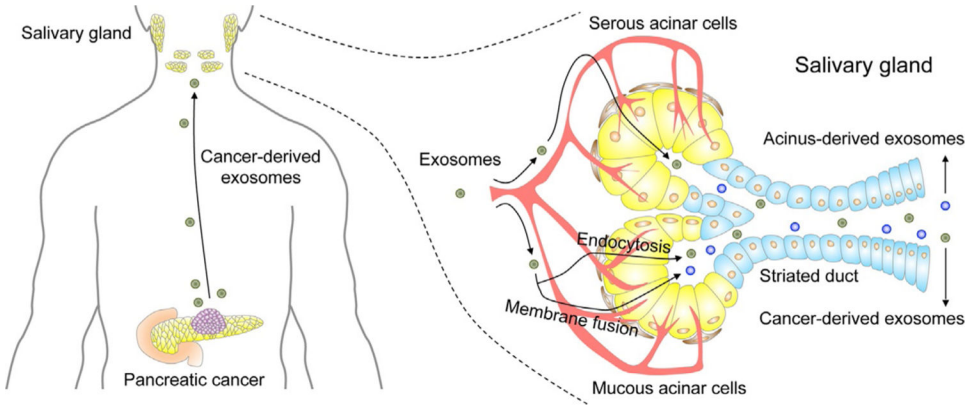

Fig. 3.

Schematic representation of the exosome-mediated transfer of cancer-derived products from distal tumor to salivary gland. Cancer-derived exosomes enter circulation and reach salivary glands. Exosome uptake at salivary gland acinar cells occurs via endocytosis or membrane fusion. Two different salivary exosomes are released into saliva. Cancer-derived exosomes are released through exocytosis, while acinus-derived exosomes are released through fusion of multivesicular bodies (MVBs) with the plasma membrane. Both types of salivary exosomes carry cargos that include cancer-derived products.

Table 1

Salivary Protein Biomarkers for Cancers

Cancer	Sample	Salivary Protein Biomarker	References
Breast cancer	Whole saliva	EGF	[85]
		c-erbB-2	[86]
		CA15–3, c-erbB-2	[87]
		VEGF, EGF, CEA	[88]
		CA6	[69]
		LRP	[89]
SCC	Whole saliva	A1BG, CFB	[22]
		M2BP, MRP14, CD59, CAT, PFN	[90]
		FB, S100, TF (transfernn), IGHG, CFL1	[16]
		ADA	[91]
		IL-8, M2BP, IL-1B	[67]
	Salivary EVs	A2M, HPA, MUC5B, LGALS3BP, IGHA1, PIP, PKM1/M2, GAPDH	[92]
Gastric cancer	Whole saliva	1472.78, 2936.49, 6556.81, 7081.17Da	[93]
		CSTB, TPI1, DMBT1, CALML3, IGH, IL1RA	[94]
Lung cancer	Whole saliva	HP, AZGP1, CALPR	[95]
	Salivary EVs	Annexin Al, A2, A3, A5, A6, All, NPRL2, CEACAM1, HIST1H4A, MUC1, PROM1, TNFAIP3	[96]
Ovarian cancer	Whole saliva	CA125	[97]

EVs, extracellular vesicles; SCC, squamous cell carcinoma.